Phenol-enriched olive oils modify paraoxonase–related variables: a Randomized, Crossover, Controlled Trial

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Abbreviations and acronyms

Ahr	aryl hydrocarbon receptor
ANOVA	analysis of variance
CCL-2	chemokine (C-C motif) ligand 2
CON	control diet
CVD	cardiovascular disease
FVOO	functional virgin olive oil enriched with its own phenolic compounds
FVOOT	functional virgin olive oil enriched with phenolic compounds from olive oil and thyme
HDL-C	HDL cholesterol
H-FVOO	high-FVOO
L-FVOO	low-FVOO
M-FVOO	medium-FVOO
MAPK	mitogen-activated protein kinases

OO	olive oil
OO-PC	olive oil phenolic compounds
PC	phenolic compounds
PON	paraoxonase
Ppar	peroxisome proliferator-activated receptor
SEC	control diet supplemented with OO-PC extract, mainly secoiridoids or hydroxytyrosol derivatives
SEC+THY	diet supplemented with secoiridoids and thyme phenols extracts
Th-PC	phenolic compounds from thyme
THY	diet supplemented with thyme phenols extract
TPC	total phenol content
VOO	virgin olive oil

Keywords: flavonoids, olive oil, phenolic compounds, PON, secoiridoids.

ABSTRACT

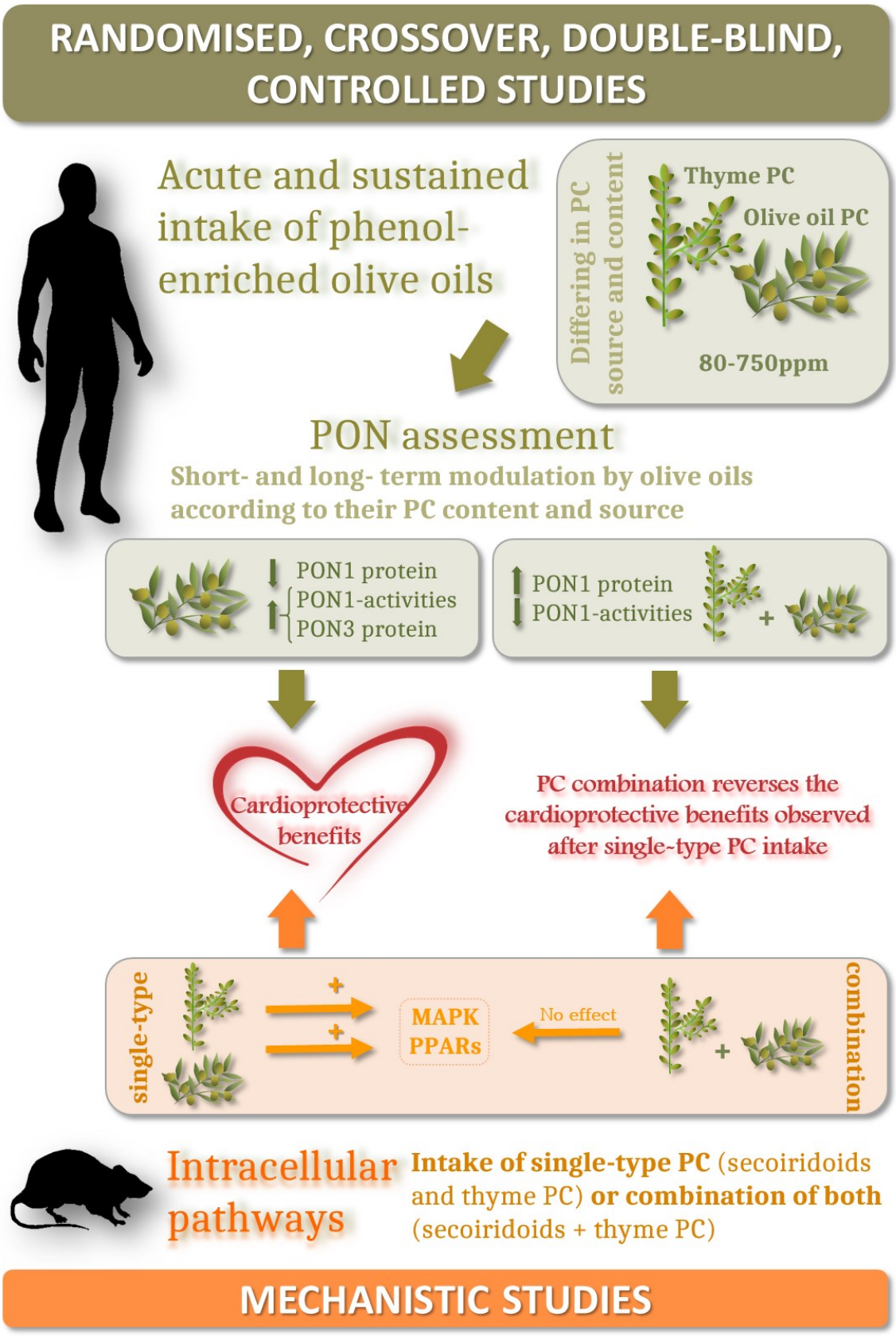
Scope: Low paraoxonase (PON)1 activities, and high PON1 and low PON3 protein levels are characteristic of cardiovascular disease. Our aim was to assess short and long-term effects of virgin olive oils (VOO), enriched with their own phenolic compounds (PC; FVOO) or with them plus complementary PC from thyme (FVOOT), on PON-related variables and the mechanisms involved.

Methods and results: Two randomized, controlled, double-blind, and crossover interventions were conducted. In an acute intake study, participants ingested three FVOOs differing in PC content. In a sustained intake study, participants ingested a control VOO and two different FVOOs with the same PC content but differing in PC source. Acute and sustained intake of VOO and FVOO decreased PON1 protein and increased PON1-associated specific activities, while FVOOT yielded opposite results. PON3 protein levels increased only after sustained consumption of VOO. Mechanistic studies performed in rat livers showed that intake of isolated PC from VOO and from thyme modulate mitogen-activated protein kinases and peroxisome proliferator-activated receptors regulating PON synthesis, while a combination of these PCs cancels such regulation.

Conclusion: The present study reveals that the intake of phenol-enriched FVOOs modulates oxidative balance by modifying PON-related variables according to PC content and source, and this modulation can be perceived as beneficial.

Two randomized, controlled, double-blind, and crossover interventions were conducted: Acute study: intake of functional virgin olive oils (FVOO) differing in phenolic compounds (PC) content; Sustained study: intake of different FVOOs (same PC content but different source). Main outcomes include that the acute and sustained consumption of olive oil-PC in humans modifies PON-related variables towards a beneficial mode, increasing PON1 protein

and decreasing its specific activities and PON3 protein. A combination of olive oil and thyme PC cancels such regulation.



1. Introduction

The paraoxonase (PON) enzyme family comprises of three members (PON1-3). In humans, PON1 and PON3 are expressed mainly in the liver and kidney, and to a lesser extent in other organs such as brain, colon, heart, kidney, lung and small intestine. They both are secreted into the blood where they associate with apolipoprotein (Apo)A-I-containing high-density lipoprotein (HDL) [1–3]. PON2 is exclusively intracellular and it is expressed in nearly all human tissues, though it is not found in circulation [4, 5]. All PON enzymes have lactonase activity, and PON1 can also hydrolyze aromatic esters and certain organophosphate compounds, such as paraoxon, having arylesterase and paraoxonase activities [2]. PON1 and PON3 exert beneficial effects on cardiovascular disease (CVD) by preventing lipoprotein oxidation, metabolizing lipid peroxides, promoting cholesterol efflux to HDL, and showing anti-inflammatory activity via inhibition of chemokine (C-C motif) ligand 2 (CCL-2) [2, 6–9]. PON1 and PON3 proteins, together with PON1-associated activities, play a crucial role in CVD and other related conditions, since decreased PON3 mass, increased PON1 mass, and decreased PON1-associated activities have been related to an increase in plaque formation and to high CVD risk [5, 7, 10–13].

Phenolic compounds (PC), naturally present in fruit and vegetables, modulate hepatic PON1 gene expression as well as hepatic and plasma activities in mice [14–16] and humans [17, 18]. In particular, hydroxytyrosol from olive oil (OO) and flavonoids from several sources have been extensively described as triggering PON1 activities in animals [15, 19, 20] and humans [18, 21, 22]. In 2011 the European Food Safety Authority (EFSA) released a claim concerning the effects of a daily intake of 5 mg of the PC hydroxytyrosol (a simple phenolic compound) and its derivatives on low-density lipoprotein (LDL) protection from oxidation [23]. Nevertheless, the phenolic content in most virgin OOs (VOO) available on the market

are low to allow the recommended consumption of hydroxytyrosol and its derivatives within the context of a balanced diet [23]. The enrichment of VOO with its own PC, or with complementary PC from other sources, has been postulated as an interesting strategy to increase the daily PC intake without increasing caloric intake [24, 25]. Thyme (*Thymus zyguis*) is the herb selected in this study for VOO flavoring, as it is one of the richest sources of flavonoids [26]. Our previous studies have reported that PC from thyme (Th-PC) enhances the bioavailability of the PC from OO (OO-PC) [27, 28], and that a thyme-enriched OO intervention improves HDL oxidative status [29] and DNA protection against oxidation [30], and exerts a cardioprotective impact on HDL particles [31–33]. However, the prospective beneficial properties of these phenol-enriched VOOs on PON enzymes remain unknown. Furthermore, most of the studies assessing PON concentrations and activities have been undertaken after sustained interventions and thus only long-term modifications have been studied. As HDL turnover is considerable, it seems safe to predict that PON enzymes might also undergo short-term modifications. As a result, postprandial studies are needed to obtain further insights regarding PC effects on PON family. Additionally, no data concerning the transcriptional effects of OO-PC on PON1 have been published to date, as most mechanistic studies are focused on PON1 regulation carried out by drugs or flavonoids [1–3, 34].

Our hypothesis is that the consumption of OO-PC may modulate PON-related variables (*i.e.* PON1 and PON3 protein levels along with PON-1 lactonase and paraoxonase activities) towards a beneficial mode, and that blending OO-PC with complementary Th-PC, might instigate synergic effects. The aim of the present work was to assess the short and long-term effects of the acute and sustained intake of different VOOs, enriched with OO-PC or with them plus complementary Th-PC, on PON-related variables. A secondary objective was to investigate the mechanisms involved in the OO-PC and Th-PC effects on such variables.

2. Materials and methods

2.1 Phenol-enriched OO preparation and composition

Different set of VOOs were prepared for each of the 2 interventions. For the acute study, three VOOs enriched with their own PC, but differing in their total phenolic content were prepared as previously described [24]: Low-functional VOO (L-FVOO: 250 ppm), Medium-functional VOO (M-FVOO: 500 ppm) and High-functional VOO (H-FVOO: 750 ppm). A VOO (80 ppm) was used as the matrix to prepare these phenol-enriched VOOs by adding a freeze-dried olive cake extract rich in the main OO-PC, mainly secoiridoids or oleuropein derivatives as the main source of hydroxytyrosol [35]. Regarding the sustained intake study, the same parental VOO used for the acute study was used as a control condition and as a matrix to prepare two functional VOOs, enriched with equal content of PC (500 ppm) but differing in the PC source. The first one was enriched with its own PCs (FVOO) while the second one (FVOOT) was enriched with its own PC (50%; mainly secoiridoid derivatives) plus complementary PC from thyme (50%; mainly flavonoids, phenolic acids, and monoterpenes) [26]. These three VOOs did not differ in fatty acid and micronutrient composition other than the phenolic content [29, 31]. The phenolic composition of the VOOs used in both studies was quantified by UPLC-MS/MS following the chromatographic method described previously [27]. The phenolic intake through the ingested dose of the VOOs used in the acute (30 mL) and sustained study (25 mL) is shown in Supporting Information Table S1.

2.2 Designs of the studies and participants' characteristics

Both studies were randomized, controlled, double-blind and crossover trials. In the acute study the short-term effects of VOO with different PC content on PON-related variables were determined. In the sustained study the long-term effects of phenol-enriched VOOs, with the same PC content but with PC from different sources, on PON-related variables were

determined. As depicted in Figure 1A, the acute study was carried out in 12 participants, considered healthy according to a physical examination and routine laboratory tests. Participants were instructed to follow a stabilization diet for 2 weeks before the first intervention and 1-week washout periods between each VOO intervention in order to avoid potential carry-over effect. In the interventions, participants ingested a single dose of 30 mL of raw VOO (L-FVOO, M-FVOO or H-FVOO). Fasting blood samples were collected at baseline and at different postprandial times (2h, 4h and 6h). The sustained study (Figure 1B) was conducted in 33 hypercholesterolemic subjects (total cholesterol>200 mg/dL). Participants ingested a daily dose of 25 mL of raw OO for 3 weeks, accordingly with the assigned sequence of intervention, preceded by 2-week washout periods with a common OO. To avoid an excessive intake of antioxidants during the clinical trial period, participants were instructed to limit the consumption of PC-rich foods. Blood samples were collected at the beginning and at the ending of each intervention, and sera and plasma EDTA samples were stored at -80ºC until their use. Glucose and lipid profile were measured in plasma EDTA, in a Cobas-Mira Plus (Roche Diagnostic System, Spain) and ApoA-I, ApoA-II, and ApoB-100 in a PENTRA-400 (ABX-Horiba Diagnostics, France) automated analyzers. The details of the participants and the protocol of both studies have been previously described [29, 33]. The present clinical trials were conducted in accordance with the Helsinki Declaration and the Good Clinical Practice for Trials on Medical Products in the European Community and International Conference of Harmonization. The subjects gave their written informed consent before their participation. Acute study was registered at ClinicalTrials.gov (Identifier: NCT01347515). Sustained study was registered at the International Standard Randomized Controlled Trial register (Identifier: ISRCTN77500181).

2.3 Experimental procedures with rats

To add insights to the underlying mechanisms involved in PON modulation, 20 male and female Wistar rats (Charles River Laboratories; Spain) weighing 300-350g were allocated to four groups: CON, control diet; SEC, control diet supplemented with OO-PC extract, mainly secoiridoids or hydroxytyrosol derivatives; THY, diet supplemented with Th-PC extract; and SEC+THY, diet supplemented with secoiridoids and Th-PC extracts. Extracts were prepared as previously described [35]. SEC and SEC+THY extracts correspond to the ones used for FVOO and FVOOT preparation, respectively. Rats ingested 5 mg of the appropriate phenolic extract/kg rat/day for 21 days. As the matrix employed in the four groups was the same, *i.e.* the commercial feed pellets Teklad Global 14% Protein Rodent Maintenance Diet (Harlan Laboratories, Santa Perpètua de Mogoda, Spain), the effects observed between groups may be entirely attributed to their different phenolic content. The dose selected was extrapolated to human dose through normalization to body surface area [36]. THY group was used to investigate the effect of an equivalent phenolic dose exclusively from thyme. Details of the diets and dosage administered are explained in Supporting Information Table S2. Rats were anesthetized with isoflurane (IsoFlo, Veterinarian Esteve, Italy) and sacrificed by intracardiac puncture. After blood collection, rats were perfused with an isotonic solution of sodium chloride 0.9% to remove the remaining blood irrigating the tissues, and their livers were excised. Sera samples were obtained and stored at -80ºC until their use. The procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEEA-Universitat de Lleida, reference 7675). Glucose and lipid profile were measured in an autoanalyzer Cobas-Mira Plus (Roche Diagnostic System, Spain).

2.4 PON3 and PON1 protein concentration

PON3 and PON1 protein concentrations were assessed in human sera by in-house ELISA with rabbit polyclonal antibodies generated against synthetic peptides with sequences specific

for mature PONs (kindly provided by Dr. Mackness, Manchester University, UK) as previously described [37].

2.5 Paraoxonase and lactonase activities

Since PON1 has esterase and lactonase activities we decided to measure the catalytic activity of PON1 using two different substrates: paraoxon (an ester) and 5-thiobutyl butyrolactone (TBBL, a synthetic lactone). Both activities were assessed in human sera as previously described [38, 39]. These activities are expressed as U/L (1U=1μmol of substrate hydrolyzed per minute), and are referred as raw activities throughout this work. Specific activities were calculated as the ratio between the activity and the corresponding concentration.

2.6 Kinases array

A kinases proteome profiler (R&D systems, USA) was used in rat liver homogenates to assess the activation of not only the three major families of Mitogen-Activated Protein Kinases (MAPK; ERK1/2, JNK1-3 and p38α/β/δ/γ) but also other related kinases that play an important role in cellular signaling pathways. 125 mg of hepatic tissue were lysed using a commercial lysis buffer (Cayman Chemical, USA) containing DTT and proteases and phosphatases inhibitors. Finally, 200μg of total protein assessed with Bradford assay, were employed to perform the array. Chemiluminescence signal was measured in Amersham Imager 600 reader and data were acquired using Image Quant TL program (GE Healthcare, Spain). CON values were set at 1 and SEC, THY and SEC+THY results are expressed as fold-increase or -decrease *vs.* CON values.

2.7 Real-Time Quantitative PCR

Gene expression of *Pon1*, *Pon3*, *Ccl-2*, *aryl hydrocarbon receptor* (*Ahr*), *peroxisome proliferator-activated* (*Ppar*)*α, δ* and *γ* were analyzed in rat liver homogenates. 30 mg of hepatic tissue were homogenized and lysed with a rotor-stator DIAX 900 homogenizer (Heidolph, Germany). Total RNA was purified, quantified and reverse transcribed to cDNA

as previously described [40]. Real-time quantitative PCR was performed in ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, USA). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method with *glyceraldehyde 3-phosphate dehydrogenase* as housekeeping gene. CON values were set at 1 and SEC, THY and SEC+THY results are expressed as fold-increase or -decrease *vs.* CON values.

2.8 Sample size and power analysis

In the acute study, a sample size of 12 individuals allows a power of at least 80% to detect a statistically significant difference between groups of 1.7 mg/dL of HDL cholesterol (HDL-C) and a SD of 1.9. In the sustained study, a sample size of 30 individuals allows a power of at least 80% power to detect a statistically significant difference between groups of 3 mg/dL of HDL-C and a SD of 1.9. In both cases, a dropout rate of 15% and a Type I error of 0.05 (2-sided) were assumed.

2.9 Statistical Analyses

Kruskal-Wallis test or 1-factor analysis of variance (ANOVA) with Bonferroni correction was used to discard differences in baseline characteristics. Carryover effect was discarded by testing a period-by-treatment interaction term in general linear models. A general linear model for repeated measures was used to assess the OOs effects within and between interventions (intra- and inter-intervention differences, respectively). In the animal studies, ANOVA with Bonferroni correction was used to assess the effects of the phenolic extracts. Pearson correlations were calculated between the variables described in results section. Statistical significance was defined as $p<0.05$ for a 2-sided test. Analyses were performed using SPSS for Windows, version 22 (IBM corp., USA).

3. Results

3.1 Characteristics of the study participants

For the acute study 13 participants were recruited, and 12 were eligible and completed the study. For the sustained study, 62 participants were recruited and 33 were eligible to be enrolled in the study. No adverse events related to OO intake were reported. Baseline characteristics of acute and sustained study participants are described in Supporting Information Tables S3 and S4, respectively. No statistical differences were found between treatment sequences.

3.2 Acute study

3.2.1 PON3 and PON1 protein levels

All OO tested showed a similar time-course trend for PON3 characterized by a decrease at 4h-time point (4.2-11%; $p<0.05$; Figure 2A) *vs.* their baseline. No statistical differences were observed among the functional VOOs tested at any time point. Regarding PON1 (Figure 2B), L-FVOO and M-FVOO decreased this protein after 2h of OO intake (5.1-6.4%; $p<0.05$), while H-FVOO promoted a trend to increase PON1 at this time point *vs.* baseline (6.8%), although without statistical significance. These different time-course trends were statistically significant at 2h when comparing L-FVOO and M-FVOO with H-FVOO ($p<0.05$). Detailed data of PON1-3 proteins are shown in Supporting Information Tables S5 and S6.

3.2.2 PON1 lactonase and paraoxonase activities

After all the functional VOOs tested, lactonase raw activity peaked at 2h-time point (3.3-7.1%) and then reached a steady state at 4h (Figure 2C). Inter-interventions changes were observed at 4h- and 6h-time points between H-FVOO and the other two functional VOO ($p<0.05$). Concerning paraoxonase raw activity, L-FVOO and M-FVOO intake prompted a mild increase at 2h-time point followed by a decrease at 4h-time point ($p<0.05$). In contrast,

H-FVOO triggered a steady drop throughout the time-frame assessed ($p<0.05$). Nevertheless, no inter-interventions changes were observed in the paraoxonase kinetics at any time point (Figure 2D). Regarding lactonase and paraoxonase specific activities, both peaked 2h after L-FVOO and M-FVOO intake, dropping to basal values at 4h-time point (Figures 2E-F; $p<0.05$). H-FVOO consumption followed different kinetic trend, as no peak was observed. When inter-intervention differences were addressed, H-FVOO showed lower paraoxonase and lactonase specific activities at 2h when compared with L-FVOO and M-FVOO, respectively ($p<0.05$). Detailed data of PON1-associated activities are shown in Supporting Information Tables S5 and S6.

3.3 Sustained study

3.3.1 PON3 and PON1 protein levels

PON3 levels increased by 5.1% after VOO intervention ($p<0.05$; Figure 3A). This increase was significant when compared with the changes observed after FVOO and FVOOT interventions ($p<0.05$). PON1 levels decreased by 10.9-12.4% after VOO and FVOO respectively, whilst increased by 4.04% after FVOOT ($p<0.05$; Figure 3B). This increase turned out to be statistically significant when compared with the changes observed after VOO and FVOO interventions ($p<0.05$).

3.3.2 PON1 lactonase and paraoxonase activities

FVOO decreased lactonase raw activity (3.39%; $p<0.05$; Figure 3C), which turned out to be significant when compared with VOO and FVOOT interventions. VOO increased and FVOOT decreased paraoxonase raw activity ($p<0.05$; Figure 3D). These changes were statistically different when compared with FVOO intervention ($p<0.05$). VOO and FVOO increased lactonase and paraoxonase specific activities to similar extent (8.1%-12%) whereas FVOOT promoted a trend to decrease these activities (9.3 and 13.7% respectively) although without statistical significance (Figures 3E-F). These decreases observed after FVOOT were

statistically different when compared with the increases observed after VOO and FVOO intake ($p<0.05$). Comprehensive data of PON levels obtained in the chronic study is shown in Supporting Information Table S7. After all OO tested, strong positive correlations were observed between the activities assessed ($p<0.05$; Supporting Information Table S8) but no between activities and PON1-3 proteins (data not shown). In addition, after VOO intake, paraoxonase specific activity directly correlated with ApoA-I determined in HDL and in plasma EDTA ($r=0.529$ $p=0.003$; $r=0.481$ $p=0.007$ respectively). Paraoxonase specific activity and ApoA-I also correlated positively after FVOO and FVOOT intake, but such correlations were weaker and statistically non-significant.

3.4 Mechanistic study: Animal model

3.4.1 Glucose and plasma lipid profile

No statistical differences were found between treatment groups (CON, SEC, THY and THY+SEC) at the end of the interventions (Supporting Information Table S9).

3.4.2 Kinases activation

MAPK activation was assessed in rat hepatic tissue, as several transcription factors regulated by this kinase family modulate PON1 expression in human hepatic cells [41, 42] and rat liver homogenates [43]. Activation of the vast majority of MAPK was observed after diet supplementation with SEC and THY extracts when compared with control diet (CON; $p<0.05$; Figure 4A). Diet supplementation with SEC+THY abolished ERK1, JNK2, JNK-PAN and p38β activation observed after SEC and THY intake ($p<0.05$). Kinases, other than MAPK, activation was also assessed (Figure 4B).

SEC and THY intake increased almost all these kinases activation to the same extent ($p<0.05$), except for p53 that was activated only by THY. SEC+THY intake activated (MKK6, MSK2, Akt pan) and inhibited (p53) some kinases compared with CON ($p<0.05$). This regulation resulted statistically significant also compared to SEC and THY (MKK3,

RSK1 and GSK-3α/β; $p<0.05$). Other kinases with no statistical changes were studied (HSP27, Akt1, Akt3, CREB, GSK-3β and TOR; data not shown).

3.4.3 Transcription factors gene expression

SEC and THY diet supplementation decreased *Pparα* to the same extent compared to CON ($p<0.05$), showing a tendency to increase *Pparδ* and *Pparγ*. No effects were observed after SEC+THY in any *Ppar* studied (Figure 5A). No changes were observed on *Ahr* gene expression in response to the intake of any of the extracts studied (data not shown). However, *Ahr* gene expression positively correlated with *Pparδ* in SEC and had a tendency to correlate with *Pon1* in CON ($r=0.953$; $p=0.047$ and $r=0.840$; $p=0.075$, respectively; Supporting Information Table S10).

3.4.4 Pon1, Pon3 and Ccl-2 gene expression

SEC intake increased *Pon3* hepatic gene expression and had a tendency to increase *Pon1 vs*. CON group ($p<0.05$), while THY intake decreased *Pon1* and *Pon3 vs.* SEC group ($p<0.05$; Figure 5B). *Pon1* gene expression correlated with *Pparγ* ($r=0.966$; $p=0.034$) and had a tendency to correlate with *Pparα* ($r=0.932$; $p=0.068$) in SEC group (Supporting Information Table S10). No statistical differences were shown in *Ccl-2* gene expression, surely due to the high inter-individual variability (data not shown).

4. Discussion

The present work confirms the hypothesis that the acute and sustained intake of phenol-enriched VOOs modulates PON-related variables according to PC source and content. On the one hand, after the acute and sustained intake of OO-PC (via VOO and FVOO intake) a decrease in PON1 protein, together with increases in PON3 protein levels and PON1-associated specific activities are reported. On the other hand, sustained intake of a mixture of OO-PC and Th-PC (via FVOOT intake) induces opposite results rather than producing synergic effects as we had hypothesized. These differences may be due to the combination of

OO-PC with Th-PC intake, rather than the sole presence of Th-PC, since mechanistic studies revealed that single-type PC modulated PON synthesis, while no effects were observed when PC were combined. To the best of our knowledge, this is the first time that a human trial has been conducted to integrate the short and long-term effects of PC consumption on PON-related variables.

In the human acute study, the time-course trend for PON1 protein and associated specific activities turn out to be different according to the content of OO-PC of the FVOO consumed. PON1 protein reached minimum and specific activities peaked 2h after L-FVOO and M-FVOO intake while different kinetics were observed with H-FVOO. Such observations are supported by the bioavailability of the OO-PC themselves, as OO-PC undergo a rapid absorption with T_{max} around 1–2h after ingestion, and plasma concentrations return to baseline values 6h after ingestion [24]. Actually, the selection of M-FVOO for the sustained-intake study was made according to previous works developed by our group. On the one hand, a pharmacokinetic study revealed that plasmatic phenolic metabolites did not show a complete linear response after 500 ppm and 750 ppm, indicating that a threshold could exist in OO phenolic absorption [24]. On the other hand, M-FVOO provided additional benefits in the endothelial function versus L-FVOO and H-FVOO, and showed a better postprandial response on several cardiovascular risk biomarkers [44].

In the sustained intake study, the present work shows that VOO and FVOO intake equally decreased PON1 protein and increased PON1-associated specific activities, irrespective of OO-PC content. Increased PON1 protein levels and decreased activities are characteristic of diseases whose underlying mechanisms comprise an impairment of oxidative stress, in particular CVD [10, 11, 45, 46], diabetes *mellitus* [47], inflammatory diseases [48, 49], cancer, and several hepatic and renal diseases [50, 51], all characterized by having dysfunctional HDL particles and increased CVD risk [2]. Furthermore, PON1 activities

positively correlate with the improvement of HDL antioxidant properties, to such an extent that PON1 activities have been proposed as new biomarkers of HDL function and CVD risk [10, 52]. Therefore, the decrease in PON1 protein and the increase in its associated activities can be perceived as being beneficial as they might be indicative of a proper oxidative balance and therefore of HDL function enhancement.

The combination of OO-PC with Th-PC via FVOOT yielded opposite effects than those observed after OO-PC intake, as FVOOT increased PON1 protein and decreased its associated specific activities. Hence, beneficial effects observed after OO-PC may be due to the secoiridoids (the main phenolic compounds of VOOs) irrespective of their content, and these effects may be reversed by Th-PC, mainly flavonoids. Our previous studies have reported that flavonoids present in FVOOT enhance cardioprotective benefits [29–33]. Similarly, pomegranate juice rich in flavonoids and phenolic acids, and extra-VOO enriched with green tea flavonoids, increase arylesterase activity in ApoE-deficient mice [16, 20] and humans [18]. Red wine flavonoids and stilbenes trigger paraoxonase activity in hypercholesterolemic hamsters [19]. The dissimilarities between the results obtained in the present work and those from other authors could be attributed to the combination of Th-PC with OO-PC, rather than the sole presence of flavonoids, as discussed further on.

PON3 protein levels increased after the sustained intake of control VOO suggesting that such VOO plays a protective role, as PON3 depletion from HDL is associated with the presence of subclinical atherosclerosis in patients with autoimmune diseases [7], chronic hepatitis, and liver cirrhosis [53]. Our results partially agree with those observed in a proteomic study carried out in the HDL from the VOHF participants, where an increase in PON3 protein was reported after the sustained intake of VOO, FVOO, and FVOOT [33].

The effects observed in PON-related variables in the present work can be translated in an enhancement of HDL functionally. Within the frame of the VOHF study, an improvement in

HDL functionality, particularly in the HDL-mediated cholesterol efflux has been observed following phenol-enriched VOOs consumption (submitted data). Additional effects related to the enhancement of HDL function after phenol-enriched VOOs have already been published by our group such as improvement of oxidative status [29], DNA protection against oxidation [30], changes in HDL size and distribution [31], and changes in HDL proteome [33]. Moreover, PON1 activities have been proposed as promising biomarkers of HDL function and CVD risk [10, 52]. Therefore, the increase in PON1-associated activities observed after the intake of phenol-enriched VOO may be indicative of HDL functionality enhancement.

In the present work, mechanistic studies were conducted in rat liver homogenates to provide insight into the intracellular pathways involved in hepatic PON synthesis modulation [1–3, 34] and to disclose whether the effects observed after FVOOT were due to the Th-PC *per se* or to their combination with OO-PC. To date, this is the first time that p38 MAPK family and MAPK-related kinase effects on PON family are reported. The intake of a single-type PC (SEC or THY groups) activated the three families of MAPKs and a vast majority of MAPK-related proteins, decreased *Pparα*, and showed a tendency to increase *Pparδ* and *Pparγ* to the same extent, as similarly described by our group in human white blood cells [54]. Interestingly, these effects were not observed after the combination of PC (SEC+THY group). However, a minor impact on PON gene expression was observed. Despite showing equal effects on *Ppars* and kinases activation, SEC increased *Pon3* and showed a tendency to increase *Pon1* expression while THY decreased both *Pon1* and *Pon3*. *Moreover, Pon1* directly correlated with *Pparγ* and *Pparα* in the SEC group. This suggests that hepatic PON1 regulation may be mediated via *PPARγ*, which in turn is mediated via ERK1/2 activation, as previously described in glitazones, statins, and pomegranate juice [34, 53, 54]. In concordance, our group has previously reported that SEC down-regulates Nuclear Factor-ƙB

activation, a transcription factor that is inhibited by *PPARγ* [30]. THY decreased hepatic *PON1* and *PON3* expression compared to SEC, despite showing equal effects on *PPAR*, MAPK and MAPK-related proteins. These data suggest that Th-PC modulates PON hepatic synthesis by means of intracellular pathways other than the PPAR family. The combination of OO-PC with Th-PC had no impact on PON hepatic synthesis, in concordance with the lack of effects on kinases and PPARs activation observed in the current study. In general terms single-type PC, but not their combination, modulated intracellular pathways that regulate hepatic PON synthesis. These results help us to understand those obtained in the human studies. That is, the combination of OO-PC and Th-PC, rather than the sole intake of Th-PC, is accountable for the effects on PON-related variables.

Several factors other than PON hepatic synthesis regulation might explain the PC effects observed in the human studies. Those include hepatic PON1 degradation and secretion to the extracellular space [43], PON1 binding to HDL and activation by ApoA-I [3, 50, 57], and PON1 redistribution to large and mature HDL [47, 58]. Concordantly, we have previously reported that FVOO decreases the small-HDL/large-HDL ratio, increases HDL particle size [31], and increases CETP activity [29], all of them indicative of an enhancement of HDL maturation. Additionally, the EUROLIVE study showed that the presence of PC metabolites in HDL subsequent to the sustained intake of OO-PC-enriched VOOs was accompanied by the increase of HDL_2 and the decrease in HDL_3 particles [59].

Taking the previously data into consideration, the present work provides first level evidence of the modulation of PON enzymes family following the acute and sustained intake of PC-enriched VOOs, according to PC source and content. Several mechanisms may be involved in PON enzymes family modulation, such as the activation of hepatic MAPK and *Ppar*s among

others. The changes in PON enzymes family observed may be translated into an improvement in the oxidative balance and therefore in the HDL functionality.

Author contributions

SF-C, A-IG-H, M-CLH, AP, and UC conceived, designed and performed the experiment. SF-C, A-IG-H, RS, and JC analyzed and interpreted the data. SF-C, RS, and JC drafted the paper. This paper is critically revised by RS, JC, M-CLH, MF, AP, LR, M-JM, OC, M-IC, and R-MV.

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Conflict of interest

There are no competing interests to declare.

5. References

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Figure legends

Figure 1. Human studies design. A) In the acute study the short-term effects of phenol-enriched VOOs differing in their PC content were assessed: L-FVOO with a low total phenol content (TPC; 250 ppm), M-FVOO with a medium TPC (500 ppm) and H-FVOO with a high TPC (750 ppm). B) In the sustained study the long-term effects of phenol-enriched VOOs differing in its composition were assessed: control VOO (80 ppm); FVOO, enriched with OO-PC (500 ppm); FVOOT, combining OO-PC (250 ppm; 50%) and Th-PC (250 ppm; 50%). PON1 and PON3 protein levels, and lactonase and paraoxonase activities were measured at the specified time-points.

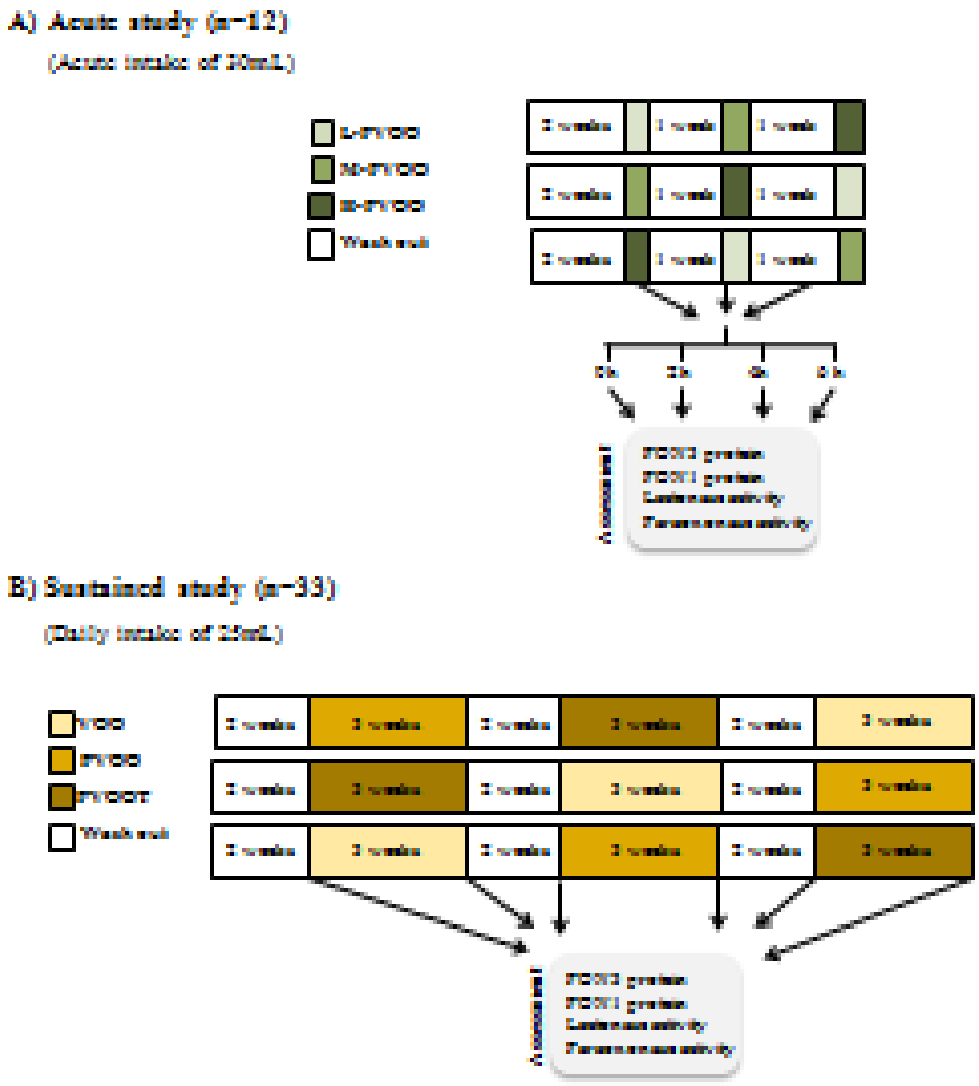


Figure 2. PON kinetics observed in the acute intake study. Participants ingested 30 mL of a phenol-enriched VOO differing in their PC content (L-FVOO with a low TPC, 250 ppm; M-FVOO with a medium TPC, 500 ppm; H-FVOO with a high TPC, 750 ppm). PON-related variables were assessed at different postprandial times. A) PON3 protein, B) PON1 protein, C) Lactonase raw activity, D) Paraoxonase raw activity, E) Lactonase specific activity, F) Paraoxonase specific activity. Results are expressed as mean ± SEM. $p<0.05$ *vs.* *L-FVOO; *vs.* †M-FVOO; *vs.* ‡H-FVOO.

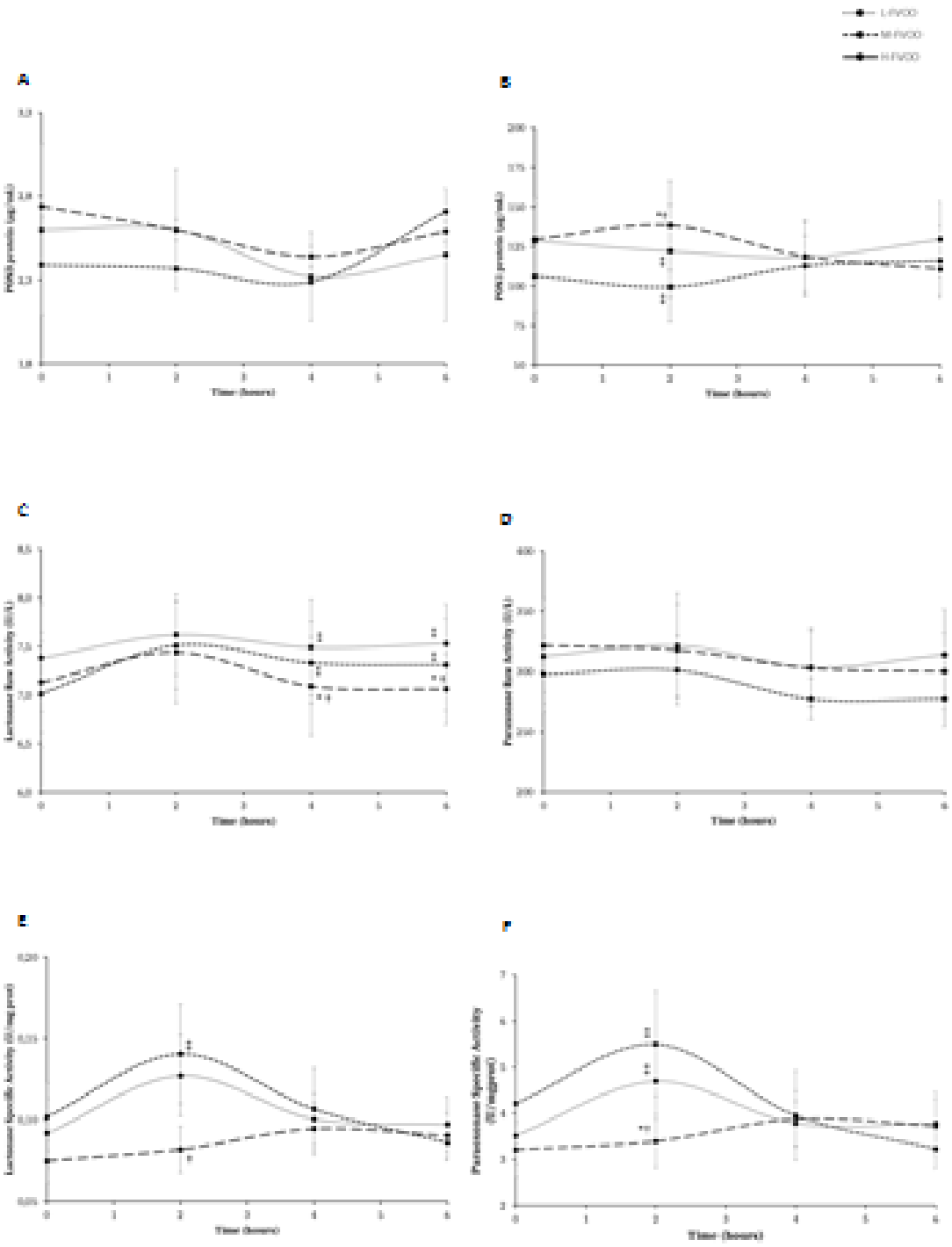


Figure 3. Changes in PON-related variables observed in the sustained study. Participants ingested 25 mL (22g) of raw OO/day for 3 weeks: control VOO (80 ppm); FVOO enriched with its own PCs (500 ppm) or FVOOT enriched with the same content of PC but differing in their source (500 ppm; 50% OO-PC and 50% Th-PC). PON-related variables were assessed in serum samples at baseline and at the end of each intervention: A) PON3 protein, B) PON1 protein, C) Lactonase raw activity, D) Paraoxonase raw activity, E) Lactonase specific activity, F) Paraoxonase specific activity. Results are expressed as mean ± SEM. *$p<0.05$ *vs.* baseline; †$p<0.05$ between interventions.

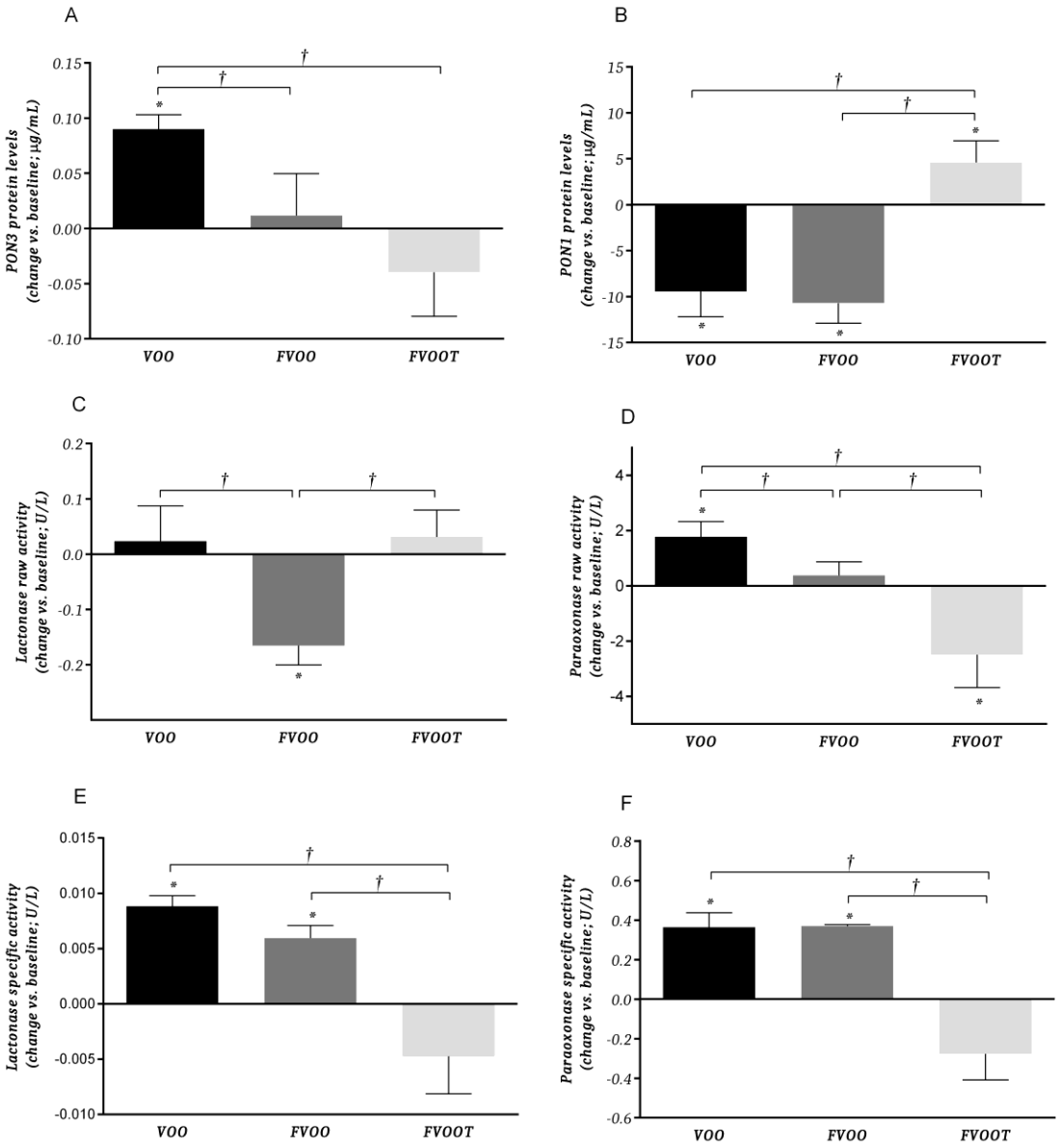


Figure 3

Figure 4. PC effects on A) MAPK and B) MAPK-related kinases activation measured in rat hepatic tissue. Rats ingested 5 mg of the appropriate phenolic extract/kg rat/day for 21 days and at the end of the study rats were anesthetized with isoflurane and sacrificed by intracardiac puncture. After blood collection, rats were perfused with an isotonic solution of sodium chloride 0.9% to remove the remaining blood irrigating the tissues, and their livers were excised. Hepatic tissue was then lysed and kinases activation were assessed in this tissue. Other kinases with no statistical changes were studied (HSP27, Akt1, Akt3, CREB, GSK-3β, and TOR; data not shown). CON values were set at 1 and SEC, THY and SEC+THY results are expressed as fold-increase or -decrease *vs.* CON values. *$p<0.05$ vs. control; †$p<0.05$ vs. SEC; ‡$p<0.05$ vs. THY.

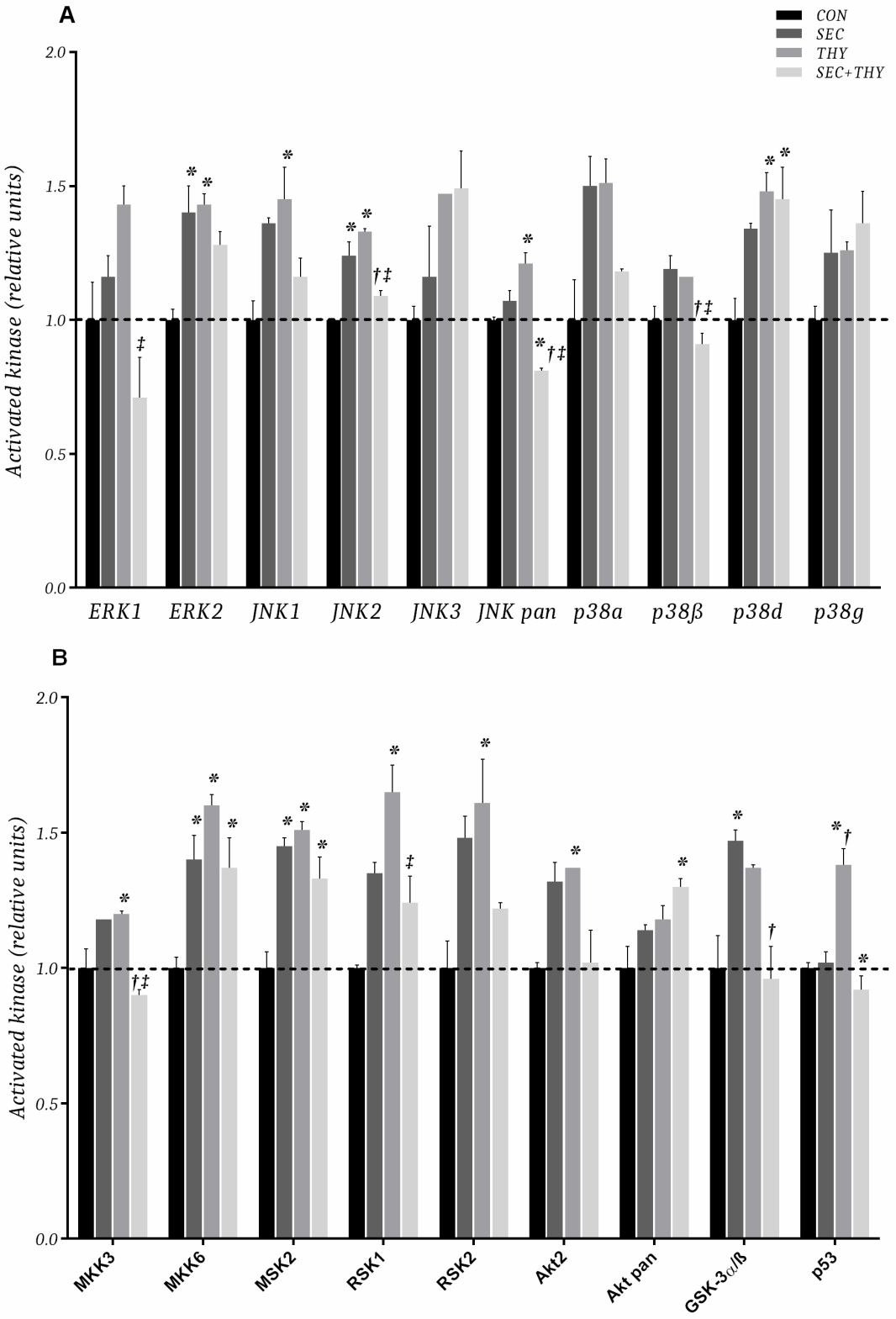
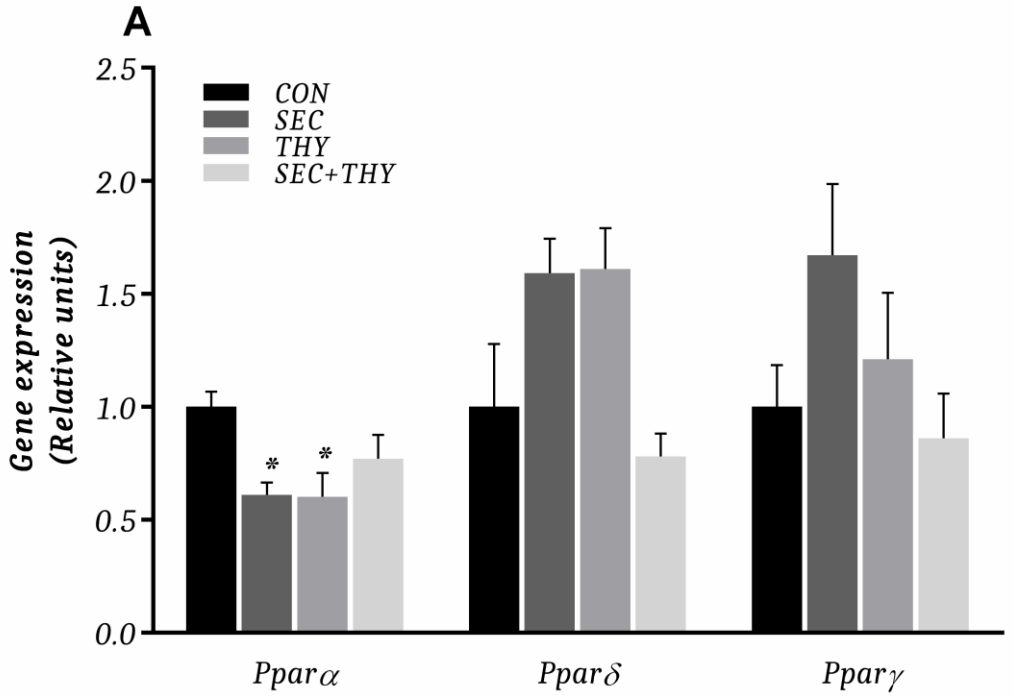


Figure 4

Figure 5. PC effects on gene expression in rat hepatic tissue. A) *Pparα, Parδ, and Pparγ*; B) *Pon3* and *Pon1*. Rats ingested 5 mg of the appropriate phenolic extract/kg rat/day for 21 days and at the end of the study rats were anesthetized with isoflurane and sacrificed by intracardiac puncture. After blood collection, rats were perfused with an isotonic solution of sodium chloride 0.9% to remove the remaining blood irrigating the tissues, and their livers were excised, homogenized and lysed to perform Real-time quantitative PCR. CON values were set at 1 and SEC, THY and SEC+THY results are expressed as fold-increase or -decrease *vs.* CON values. *$p<0.05$ vs. control; †$p<0.05$ vs. SEC.



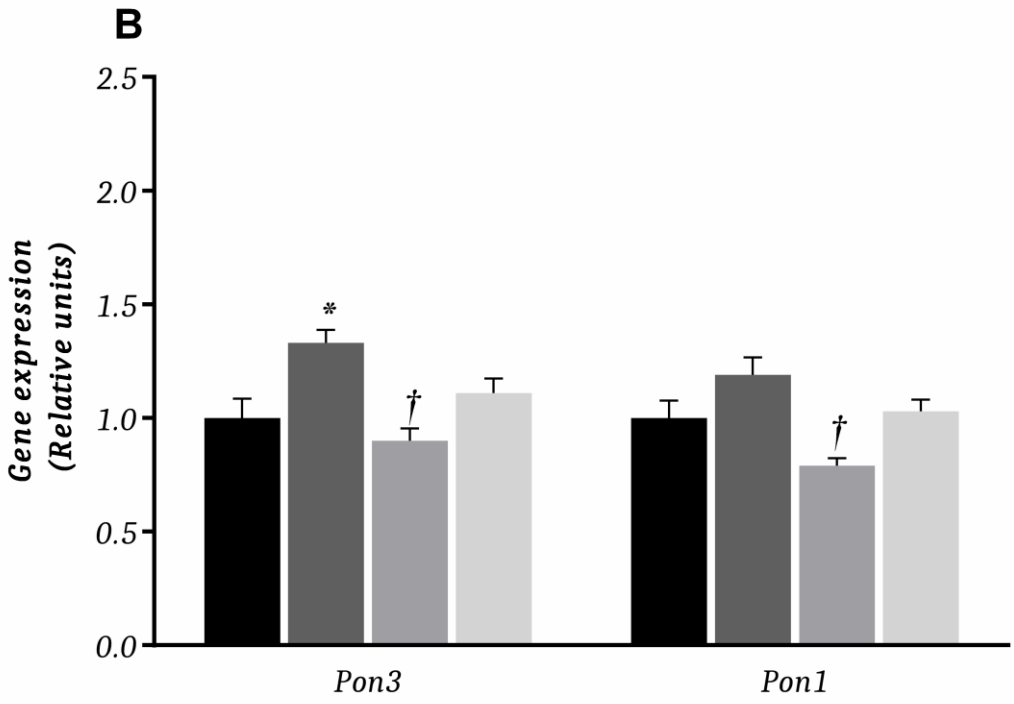


Figure 5